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## The human CD38 monoclonal antibody daratumumab shows anti-tumor activity and hampers leukemia-microenvironment interactions in chronic lymphocytic leukemia

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### Abstract

**Purpose**—To establish a proof-of-concept for the efficacy of the anti-CD38 antibody daratumumab in the poor prognosis CD38<sup>+</sup> CLL subtype.

**Experimental design**—The mechanism of action of daratumumab was assessed in CLL primary cells and cell lines using peripheral blood mononuclear cells to analyze antibody-dependent cell cytotoxicity (ADCC), murine and human macrophages to study antibody-dependent cell phagocytosis (ADCP) or human serum to analyze complement-dependent cytotoxicity (CDC). The effect of daratumumab on CLL cell migration and adhesion to extracellular matrix was characterized. Daratumumab activity was validated in two *in vivo* models.

**Results**—Daratumumab demonstrated efficient lysis of patient-derived CLL cells and cell lines by ADCC *in vitro* and ADCP both *in vitro* and *in vivo*, while exhibited negligible CDC in these cells. To demonstrate the therapeutic effect of daratumumab in CLL, we generated a disseminated CLL mouse model with the CD38<sup>+</sup> MEC2 cell line and CLL patient derived xenografts (CLL-PDX). Daratumumab significantly prolonged overall survival of MEC2 mice, completely

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### CONFLICT- OF-INTEREST DISCLOSURE

Parul Doshi is a Janssen employee. Paul W.H.I. Parren and Jeroen Lammerts van Bueren are Genmab employees and own warrants and/or stocks. Patricia Pérez-Galán has received research funding from Genmab. The remaining authors declare no competing financial interests.

eliminated cells from the infiltrated organs and significantly reduced disease burden in the spleen of CLL-PDX. The effect of daratumumab on patient-derived CLL cell dissemination was demonstrated *in vitro* by its effect on CXCL12-induced migration and *in vivo* by interfering with CLL cell homing to spleen in NSG mice. Daratumumab also reduced adhesion of CLL cells to VCAM-1, accompanied by down-regulation of the matrix metalloproteinase MMP9.

**Conclusions**—These unique and substantial effects of daratumumab on CLL viability and dissemination support the investigation of its use in a clinical setting of CLL.

### Keywords

CLL; Daratumumab; ADCC; ADCP; migration; adhesion; MMP9

## INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and is characterized by progressive accumulation of non-functional, apoptosis-resistant mature B-cells in peripheral blood (PB), bone marrow (BM), and lymphoid tissues (1;2). The majority of the tumor cells in the blood are resting. However, heavy-water experiments have shown that CLL contains a small fraction of actively proliferating cells, with approximately 2% of cells newly generated each day (3). This proliferation occurs in specific structures known as proliferation centers localized in the lymph nodes (LN) and in the BM. Thus, CLL is considered a disease characterized by a dynamic balance between cells circulating in the blood and cells located in permissive niches in lymphoid organs (1;2). The former are primarily mature-looking small lymphocytes resistant to apoptosis whereas the latter are composed by lymphocytes that undergo either proliferation or apoptosis depending on the microenvironment.

CD38 was first reported to be associated with inferior outcome by Damle *et al* in 1999 (4), and confirmed later as a prognostic factor independent of *IGHV* mutation status (5). Patten *et al* demonstrated that CD38 expression in CLL is dynamic and changes as a result of contact with activated CD4<sup>+</sup> T cells in proliferation centers, being CD38 specifically expressed on cells that are primed to proliferate in the LN (6). As a consequence, the expression of CD38 on CLL differs among lymphoid compartments, being higher in BM and LN compared to PB (7;8) and in the proliferating fraction of the tumor (9). The functional importance of CD38 in CLL extends beyond proliferation, as it appears to be linked to the tyrosine kinase ZAP-70 and characterizes CLL cells with high migratory potential (10). CD38 cooperates with CXCR4-induced migration (11) and sustains BCR-mediated signaling (12). Finally, a role of CD38 in adhesion and tissue invasion was recently recognized. CD38 forms a macromolecular complex with the integrin CD49d and the matrix metalloproteinase MMP9, enhancing CD49d-mediated cell adhesion as well as MMP9 expression and activity (13-15). This is of key relevance because CD49d surface expression strongly correlates with overall survival in CLL (16). All these properties make CD38 an attractive target for antibody therapy in CLL and other CD38<sup>+</sup> hematologic malignancies such as multiple myeloma (MM) (17), non-Hodgkin's Lymphoma (NHL), and B- and T-acute lymphoblastic leukemia.

The human anti-CD38 antibody daratumumab (DARA) has progressed to phase III clinical trials in patients with MM. DARA is a human IgG1 therapeutic monoclonal antibody (mAb) that binds to CD38. In 2015, the US FDA has approved DARA for MM patients, who have received at least three prior lines of therapy including a proteasome inhibitor and an immunomodulatory agent, or patients double refractory to these agents. Approval was based on two phase 2 studies of DARA monotherapy (16 mg/kg) in heavily treated patients (18;19). A pooled analysis of these studies revealed an overall response rate of 31%, including responses that deepened over time, and median overall survival of 19.9 months. DARA induces killing of tumor cells, mainly *via* complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) (20) and antibody-dependent cellular phagocytosis (ADCP) by macrophages (m $\phi$ ) (21) in MM and Burkitt lymphoma (BL) cell lines. In addition, DARA induces apoptosis upon secondary cross-linking (22). Recent studies have revealed previously unknown immunomodulatory effects of DARA where CD38-expressing immunosuppressive regulatory T and B cells, and myeloid-derived suppressor cells are highly sensitive to DARA treatment (23). It has also been shown that DARA can modulate the enzymatic activity of CD38 and potentially may lead to a reduction in immunosuppressive adenosine levels (24;25). This shift away from an immunosuppressive environment may lead to the generation of protective immune responses. Indeed, a concomitant increase of helper and cytotoxic T-cell absolute cell counts and production of IFN $\gamma$  in response to viral peptides was observed. Additionally, an increase in T-cell clonality in subjects who responded to DARA versus subjects who did not respond was observed indicating an improved adaptive immune response (23).

Two additional anti-CD38 antibodies have also entered clinical trials for MM and other CD38<sup>+</sup> hematologic malignancies, MOR202 (26) and isatuximab (SAR650984) (27), that are being tested alone and in combination with standard therapy.

The aim of this study was to evaluate the cytotoxic effect of DARA on CLL cells *via* CDC, ADCC and ADCP, as well as its effect on tumor cell-microenvironment interactions, using patient-derived CLL cells and CLL cell lines in *in vitro* and *in vivo* settings.

## MATERIALS AND METHODS

### Cell lines and patient samples

Primary tumor cells from 18 CLL patients (see clinical characteristics in Table 1), diagnosed according to the World Health Organization (WHO) classification criteria were used. Written informed consents of the patients were granted following the guidelines of the Hospital Clínic Ethic Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from peripheral blood by gradient centrifugation on Ficoll (GE Healthcare) and used fresh or cryopreserved in liquid nitrogen in RPMI 1640 containing 10% DMSO (Sigma-Aldrich) and 60% heat-inactivated fetal bovine serum (FBS; Life Technologies) and maintained within the Hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). The Prolymphocytic Leukemia (PLL) cell lines, MEC1, MEC2 and JVM13 as well as the Burkitt's lymphoma Daudi cell line were obtained from DSMZ. CLL primary samples and cell lines were cultured in RPMI 1640 or IMDM supplemented with 10% FBS, 2 mM L-glutamine, 50  $\mu$ g/mL penicillin/streptomycin (Life

Technologies) and were maintained in a humidified atmosphere at 37°C containing 5% CO<sub>2</sub>. Normocin (100 µg/mL) (Invivo Gen) was added to the cell line cultures to prevent Mycoplasma contamination in cell lines that were routinely tested for *Mycoplasma* infection by PCR. The identity of all cell lines was verified by using AmpFISTR identifier kit (Life Technologies).

### Therapeutic and CRPs blocking antibodies

A human IgG1 targeting CD38 (daratumumab, DARA) was generated by immunization in a HuMAb mouse (20). The human mAb IgG1-b12, specific for the HIV-1 gp120 envelope glycoprotein (28) was included in all experiments as an isotype control mAb. Both antibodies were provided by Genmab.

Anti-CD46 (clone TRA-2-10, Biolegend), anti-CD55 (clone 1C6, Hycult Biotech) and anti-CD59 (clone YTH 53.1, AbD Serotech) antibodies were used to block complement regulatory proteins (CRPs).

### Antibody-dependent cellular cytotoxicity (ADCC)

Target cells were labeled with 1 µM Calcein-AM (Life Technologies) for 30 minutes (min) at 37°C. Afterwards, cells were washed thrice with PBS, plated in triplicate at  $1 \times 10^4$  cells/well in 96-well round bottom plates, and pre-incubated (room temperature for 15 min) with 10-fold serial dilutions of either isotype control (IgG1-b12) or DARA (range: 1 to 0.0001 µg/mL) in RPMI 1640. DARA doses for *in vitro* studies were previously established (20). Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, fresh human PBMC were added at an effector:target (E:T) ratio of 50:1, optimized in a previous report (20) and cells were incubated for 4 hours (hr) at 37°C. The plates were centrifuged, supernatant transferred into black plates (Thermo Scientific) and fluorescence was measured in a Synergy spectrophotometer (Bio-Tek) (excitation filter:  $485 \pm 20$  nm; band-pass filter:  $530 \pm 20$  nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\text{specific lysis} = 100 \times \frac{\text{experimental release (RFU)} - \text{spontaneous release (RFU)}}{\text{maximal release (RFU)} - \text{spontaneous release (RFU)}}$$

### Antibody-dependent cellular phagocytosis (ADCP)

Macrophages (mφ) were generated from monocytes isolated from bone marrow of the hind legs of female SCID mice (C.B-17/Icr-Prkdc<sup>scid</sup>/Crl) (Janvier Labs) by flushing the femurs. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin/streptomycin, and 50 U/ml M-CSF (Cell Guidance), and the culture medium was renewed every 3 days. On day 7, mφ were detached with 0.1% trypsin-EDTA and characterized by flow cytometry (CD11b<sup>+</sup>, F4/80<sup>+</sup>) (mouse antibodies obtained from eBiosciences and Invitrogen, respectively). The mφ were seeded at  $2.5 \times 10^5$  cells per well into non-tissue cultured treated 24-well plates and allowed to adhere overnight. Target cells (primary CLL and cell lines) were labeled with 0.01 µM Calcein-AM and added to the

mφ at an E:T ratio of 1:1 in the presence of a fixed mAb concentration of 1 µg/mL. After 4hr of incubation, the non-phagocytosed target cells were collected. The mφ were detached with 0.1% trypsin-EDTA, added to the non-phagocytosed target cells and stained for F4/80 expression. The amount of remaining target cells (calcein<sup>+</sup> F4/80<sup>-</sup>) was determined on an Attune acoustic cytometer, and the percentage of killed target cells in the presence of DARA compared to isotype control was calculated using the following formula:

$$\% \text{ eliminated target cells} = 100 - \left[ 100 \times \frac{\text{remaining target cells after DARA treatment}}{\text{remaining target cells after isotype control treatment}} \right]$$

### ***In vivo* phagocytosis assay**

*In vivo* phagocytosis assay was carried out as described by Overdijk et al (21). SCID beige mice (CB17.CG-PRKDC-LYST/CR, Charles River Laboratories) which lack NK cells, were inoculated with primary CLL cells or MEC2 cells (2×10<sup>7</sup> cells per mouse) into their peritoneal cavity, following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. Mice were randomly assigned into cohorts of three to five mice and received one intraperitoneal (i.p.) injection of 20 mg/kg of DARA or isotype control. 48 hrs later, mice were sacrificed and peritoneal lavage (PL) done by injecting the cavity with 5 mL of cold PBS. Total recovery of the peritoneal cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies (provided by Invitrogen and BD-Pharmingen, respectively). The relative percentage of remaining CLL cells from DARA-treated mice was derived from the isotype control group, which was set at 100%.

$$\% \text{ remaining target cells} = 100 \times \frac{\text{remaining target cells after DARA treatment}}{\text{remaining target cells after isotype control treatment}}$$

### **Complement-dependent cytotoxicity (CDC)**

Target cells were labeled with 1µM Calcein-AM (Life Technologies) for 30 min at 37°C. Afterwards, cells were washed thrice with PBS, plated in triplicate at 1×10<sup>5</sup> cells/well in 96-well round bottom plates, and preincubated (room temperature (29), 15 min) with 10-fold serial dilutions of either isotype control (IgG1-b12) or DARA (range: 10 to 0.01 µg/mL) in RPMI 1640. Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, 10% normal human AB serum was added and incubated for 45 min at 37°C. The plates were centrifuged, supernatants transferred into black plates (Thermo Scientific) and fluorescence measured in a Synergy spectrophotometer (Bio-Tek) (excitation filter: 485 ± 20 nm; band-pass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\text{specific lysis} = 100 \times \frac{\text{experimental release (RFU)} - \text{spontaneous release (RFU)}}{\text{maximal release (RFU)} - \text{spontaneous release (RFU)}}$$



### ***In vivo* homing**

Homing experiment was done as previously described by Vaisitti *et al* (11). Briefly, NOD/SCID gamma null (NSG) mice (bred in-house, animal facility, University of Barcelona) were randomly assigned into cohorts of four mice and pre-treated i.p. with 10 mg/kg of DARA, isotype control or anti-CXCR4 (R&D Systems). 24 hr later, mice were inoculated with fresh primary CLL ( $2 \times 10^7$  cells per mouse) *via* the tail vein following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. Mice were sacrificed 24 hr after tumor cell inoculation; PB, spleen and BM were recovered and the presence of tumor cells evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

### **Systemic MEC2 xenograft mouse model**

SCID mice were preconditioned with 25 mg/kg of busulfan 24hr before inoculation *via* tail vein of MEC2 cells ( $10^7$  cells per mouse), following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. One week later, mice were randomly assigned into cohorts of 6-7 mice. A saturating loading dose of 20 mg/kg DARA or isotype control i.p. was given on day 7 and thereafter 10 mg/kg weekly for 3 weeks. Mice were sacrificed if they lost 15-20% of weight and/or showed signs of disease. Survival studies were extended up to day 90 when the study was terminated. The presence of tumor cells was evaluated first macroscopically and then by flow cytometry. Cells from infiltrated organs were obtained by tissue homogenization. BM cells were obtained after flushing the femoral and tibia bones with RPMI 1640 media. These samples were filtered through 70  $\mu$ m nylon sieves (BD Falcon). Erythrocytes were lysed using ACK buffer (Quality Biological Inc.). The cells were labeled with huCD45/CD19/CD5 antibodies and analyzed by flow cytometry. Organ samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin fixed and embedded in paraffin. Tissue sections were stained with H&E and CD19 (Dako) antibody and evaluated by Cell B Basic Imaging Software (Olympus).

### **CLL-Patient derived mouse xenograft (CLL-PDX)**

On day 1, NSG mice were inoculated *iv* with fresh PBMCs from CLL ( $2 \times 10^7$  cells/mouse). On day 2, mice were randomly assigned to two groups (3-4 mice per group) and dosed i.p. with 20mg/kg of DARA or control isotype. Mice were sacrificed on day 5, PB, spleen and BM were recovered and the presence of tumor cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

### **Statistical analysis**

Unpaired and paired *T-tests* or one-way ANOVA were used to assess statistical differences between groups by means of Graph Pad-Prism software 4.0. For Kaplan Meier survival curves SPSS19 software was used.

## RESULTS

### Daratumumab induces ADCC

Ab-dependent killing via ADCC by Fc $\gamma$ R-bearing effector cells accounts for the anti-tumor activity of DARA in models of MM and BL (20;30). The ability of DARA to induce ADCC on CLL cells was assessed by calcein-AM release assay using PBMCs from healthy donors as a source of effector cells (mainly NK cells and monocytes). CLL cell lines and primary cells were treated with increasing concentrations of DARA or isotype control. DARA induced significant cell lysis starting at doses as low as 0.01 $\mu$ g/mL in CD38<sup>+</sup> CLL cell lines (Figure 1A), and at 0.001 $\mu$ g/mL in primary CLL cells (Figure 1B), reaching its maximum killing activity at 0.1-1 $\mu$ g/mL (mean $\pm$ SD=31.9 $\pm$ 11.6%). In contrast, the isotype control antibody did not induce significant cell lysis, tested at the maximum concentration of 1 $\mu$ g/mL (mean $\pm$ SD=12.4 $\pm$ 11.6%) (Figure 1B). No ADCC induction was detected in CD38<sup>-</sup> CLL cases (Table 1: CLL7, CLL11 and MEC1 cell line). A summary of ADCC induction in CD38<sup>+</sup> vs CD38<sup>-</sup> CLL primary cases is shown in Figure 1C. The degree of ADCC induction did not correlate with CD38 sABC for CLL cell lines and primary cells ( $r^2=0.088$ ) (Figure 1D). Altogether, these data indicates that ADCC constitutes a mechanism of DARA activity in CD38<sup>+</sup> CLL cells, but the extent of ADCC does not strictly correlate with CD38 expression.

### Daratumumab promotes CLL cell clearance by phagocytosis *in vitro* and *in vivo*

Recent results indicate that antibody-dependent cellular phagocytosis (ADCP) is a potent mechanism of action for DARA (21). We explored ADCP of CLL both *in vitro* and *in vivo*. To assess ADCP *in vitro* macrophages were generated from BM mouse monocytes stimulated with M-CSF. DARA induced ADCP in primary CLL cells (mean $\pm$ SD= 23 $\pm$ 4%) (Figure 2A). Representative flow cytometry profiles of CLL cells and macrophages after DARA treatment are depicted for CLL1 (Figure 2B). As observed for ADCC, phagocytosis was specimen-dependent and not strictly related to CD38 expression levels.

We next demonstrated the occurrence of ADCP *in vivo*. SCID *beige* mice, devoid of NK cells but with active macrophages were inoculated i.p. with primary CLL cells or the MEC2 cell line as described in materials and methods. As shown in Figure 2C, the percentage of remaining viable CLL cells after DARA treatment was significantly reduced (mean $\pm$ SD= 46 $\pm$ 5%;  $p<0.05$ , unpaired t-test) compared to the isotype control group at 100%. Remarkably, this decrease in cell number was detectable as early as 2hrs after DARA administration (data not shown). Figure 2D represents flow cytometry profiles showing the number of CLL cells (huCD45<sup>+</sup>/CD19<sup>+</sup>/CD5<sup>+</sup>) recovered from the intraperitoneal cavity after isotype control or DARA treatment. Taken together, these results demonstrate that ADCP may contribute to DARA anti-tumor activity against CLL cells both in *in vitro* and *in vivo* settings.

### Daratumumab induces limited CDC of CLL cells

DARA was selected from a panel of human antibodies for its broad-spectrum killing activity against hematological cell lines. DARA was particularly differentiated for its potent CDC activity (20). We evaluated DARA-induced CDC activity in a panel of CLL primary cells



and cell lines (Tables 1 and S1). In the majority of primary CLL samples, CD38<sup>+</sup> PLL cell lines (MEC2 and JVM13) and in CD38<sup>-</sup> cell line (MEC1), DARA did not induce significant cell death in the presence of normal human serum (10%). In five out of eighteen primary CLL cells, DARA induced just over 10% CDC (range: 10.4-25.6%). This limited CDC induction was not increased in the presence of higher human serum concentrations (data not shown). To explain the poor induction of CDC, we assessed the expression of complement regulatory proteins (CRPs) and the number of CD38 molecules/cell (CD38 sABC) on CLL cells. High expression of the CRPs: CD46, CD55, CD59 (mean±SD= 94±3 %; 92±4%; 92±8%, respectively) was detected by flow cytometry in all CLL cell lines and primary cases, while only CD46 (88%) was highly expressed in the BL cell line Daudi (Tables 1 and S1) which was used as a positive control for CDC. Blocking antibodies against these CRPs, probed to increase CDC induction by DARA in Daudi, while no effect was observed in CLL cells. However, blockade of CRPs increased CDC induced by the anti-CD20 antibodies rituximab (RITUX) or ofatumumab (OFA) in some CLL cases (Figure S1).

CD38 expression in CD38<sup>+</sup> CLL primary tumor samples (mean sABC±SD=1053±677) was lower than in CD38<sup>+</sup> CLL cell lines (mean sABC±SD=25,024±6,031), which was roughly ten-fold below that detected in Daudi (mean sABC±SD=292,131). A summary of Mean Fluorescence Ratio (MFIR) for CD38 and CRPs in CLL cells and cell lines is included in supplemental Table S2. Previous results in MM by Nijhof and cols have demonstrated that all-trans retinoic acid (ATRA) increases CD38 expression. We have analyzed this possibility in CLL. CLL cells (n=6) were pretreated with ATRA or left untreated for 48h. CD38 expression was analyzed subsequently on these CLL cells and challenged to CDC assay. As shown in Figure S2A, CD38 MFI of CD19<sup>+</sup>CD5<sup>+</sup> cells, was significantly (p<0.05) increased after ATRA treatment (average increase 30%), in a similar proportion than that shown for MM patient samples. A representative example is shown (Figure S2B). However, no CDC induction by DARA was observed in the cases analyzed (Figure S2C), indicating that this increase in CD38 expression was not sufficient to engage CDC.

In conclusion, these results indicate that DARA did not induce significant CDC in either CLL cell lines or primary CLL cells, and is probably due to high expression of CRPs and insufficient CD38 expression.

### **Daratumumab interferes with *in vitro* migration and *in vivo* homing**

Homing of CLL cells to secondary lymphoid organs is mainly coordinated by the CXCL12/CXCR4 axis (31). Using CLL primary cells and a xenograft mouse model, Vaisitti *et al.* demonstrated that CD38 synergizes with the CXCR4 signaling pathway and controls chemotaxis/homing of CLL cells through a close interaction between CD38 and CXCR4 in the membrane (11). Following this line of investigation, the effect of DARA on CLL cell migration was evaluated using a CXCL12 gradient. An anti-CXCR4 antibody was used as a positive control of migration blockade. In CD38<sup>+</sup> CLL cells, DARA inhibited CXCL12-mediated migration up to 70% (mean±SD= 44 ±16%; p<0.01; n=5), which was comparable to anti-CXCR4 treatment (Figure 3A). These results are in agreement with that previous report using the blocking anti-CD38 antibody SUN-4B7(11). We next examined DARA-mediated signaling following CXCR4-CXCL12 interaction. The immediate early effect of

stimulation for migration is the activation of ERK1/2 (11). Phosphorylation of ERK1/2 in CLL tumor cells occurred shortly after CXCR4-CXCL12 ligation and peaked at five minutes after CXCL12 addition. Treatment with DARA reduced ERK activation by CXCR4-CXCL12 in CLL1 and CLL12 while the ERK inhibition was less pronounced in CLL3 (Figure 3B), illustrating heterogeneity in primary tumor cells.

We then validated these *in vitro* migration results using the *in vivo* homing mouse model described previously by Vaisitti *et al* (11). Using NSG mice, which lack NK cells and active macrophages, we analyzed the effect of DARA on primary CLL cell migration from PB to BM and spleen. In this model, NSG mice were pretreated (day 0) with DARA, isotype control or anti-CXCR4, followed by fresh CLL cell inoculation on day 1. PB, BM and spleen cells were isolated on day 2 and CLL cells were identified as CD45<sup>+</sup>/CD19<sup>+</sup>/CD5<sup>+</sup>. Representative flow cytometry profiles from a mouse spleen are shown in figure 3C. Cell enumeration showed that CLL cells rapidly move from PB and mainly migrated to the spleen and that DARA significantly reduced this migration (55% inhibition on average,  $p<0.05$ ) (Figure 3D). Migration of CLL cells to BM was limited and was not affected by pretreatment of mice with DARA (data not shown). In conclusion, *in vivo* and *in vitro* results suggest that DARA hampers dissemination of CLL cells to secondary lymphoid organs.

### Daratumumab inhibits CD49d mediated CLL cell adhesion by reducing MMP9 levels

In addition to migration, CD38 also plays a key role in cell adhesion through physical interaction with the integrin CD49d/CD29 ( $\alpha 4\beta 1$  integrin) (15) which is the strongest flow cytometry marker associated with poor prognosis in CLL together with *IGVH* mutational status (16) and MMP9 (13). In addition, the expression of CD38 correlates with that of CD49d. We analyzed the effect of DARA on CD49d/CD29-mediated adhesion of CLL cells to vascular-cell adhesion molecule-1 (VCAM-1), an essential component of extracellular matrix. As shown in Figures 4A and 4B, when compared to isotype control antibody, DARA significantly impeded the adhesion to VCAM-1 of CLL primary cells ( $n=4$ ) and MEC2 cell line (mean $\pm$  SD=38 $\pm$  11%,  $p<0.01$ ), with no significant differences with anti-CD49d blocking antibody used here as a positive control (mean $\pm$  SD=49 $\pm$  30 %).

Moreover, CD38-CD49d complex also recruits the matrix metalloproteinase MMP9 leading to the up-regulation and activation of this metalloproteinase (13-15). Thus, we investigated the effect of DARA on MMP9 expression, by analyzing the variations in *MMP9* transcripts levels in CD38<sup>+</sup>CD49d<sup>+</sup> CLL cells. As depicted in Figure 4C, CLL cell adhesion to VCAM increased *MMP9* mRNA levels (mean $\pm$ SD=2 $\pm$ 1), and DARA completely abrogated both constitutive ( $p<0.01$ ) and VCAM-induced ( $p<0.05$ ) MMP9 expression. Altogether, these results demonstrate that DARA counteracts VCAM-1-mediated adhesion of CLL cells and induces the transcriptional down-regulation of *MMP9*.

### Daratumumab prolongs overall survival in a systemic CLL mouse model and reduces tumor burden in CLL-PDX

We successfully established a systemic MEC2 model by intravenous cell inoculation in busulfan-preconditioned SCID mice, which retain NKs and macrophages that can function

as effectors for DARA activity. Previous attempts to establish a MEC2 mouse model failed using subcutaneous cell inoculation in nude mice (32).

One week after cell inoculation, mice were randomly assigned into two groups and were administered a total of four doses of DARA or isotype control following a weekly schedule (20/10/10/10 mg/kg). These doses were chosen based on dose escalation studies in MM (33). The isotype control-treated mice started to show signs of disease (mainly weight loss >20% and rough hair) starting at day thirty-two post cell inoculation (Figures 5A-B), and all mice in the control group were sacrificed by day 40. These mice showed systemic dissemination of disease in lung, kidney, ovaries, parathyroid glands and enlarged lymph nodes (identified as CD45<sup>+</sup>/CD19<sup>+</sup>, Figure 5C-D and Figure S3), resembling aggressive CLL. Of note, a similar disseminated CLL mouse model was described by Bertilaccio *et al* using the CD38-PLL cell line MEC1 in Rag2<sup>-/-</sup>γ<sup>-/-</sup> mice, and the authors demonstrated its value as a tool to assess the efficacy of chemotherapeutic agents(34). In several mice, BM and spleen infiltration was also observed (data not shown). In contrast, in the DARA-treated group, only one mouse harbored signs of illness and required euthanasia at day 41, while the remaining animals survived and did not develop life-threatening symptoms up to day 90, when the experiment was terminated (Figures 5A-B). By this day, the antibody concentrations in the serum of DARA-treated animals had dropped to 1.5 µg/mL (Figure S4). Interestingly, in this group of mice no MEC2 cells were found by flow cytometry or IHC in the commonly infiltrated organs like lung and kidney, contrasting with the remarkable predominance of malignant, human CD19<sup>+</sup> cells observed in these secondary sites in control isotype-treated mice (Figure 5C-D), suggesting that these mice may be free of disease. These data suggest a strong anti-tumor activity and long-term survival of DARA-treated mice in this model.

In order to develop a mouse model closer to CLL biology, we established a short time CLL-PDX model using NSG mice, needed to avoid CLL clearance by mouse NKs. To provide this mouse model with FcγR-bearing effector cells, we selected CLL cases enriched in NKs and monocytes (Figure 5E). Fresh PBMCs from these patients were *iv* inoculated (day 1) and treated the following day (day 2) with DARA or control isotype. On day 5 mice were sacrificed and cells recovered from PB, BM and spleen. As described previously, cells mainly homed to the spleen, where a significant (\*p<0.05) decrease of CLL cells was found in the spleen of DARA-treated group. No significant differences were found in BM or PB resident CLL cells between the two groups (Figure S5A). Off note, when the experiment was performed with a CLL sample with reduced numbers of effectors, the anti-tumoral effect of DARA was diminished (Figure S5B-C)

## DISCUSSION

Targeted immunotherapy with mAbs has become the standard of care for successful treatment of many forms of cancer. In CLL, anti-CD20 antibodies (rituximab, ofatumumab and obinutuzumab), have demonstrated therapeutic benefit, alone and in combination with chemotherapy (35;36). Identification of new targets with a broader expression spectrum and potential for distinctive mechanisms could yield novel antibody therapeutics for a wider range of hematologic malignancies.

In the last years, CD38 has gained momentum as a novel therapeutic target for patients with hematologic malignancies, namely MM (37;38), CLL and non-Hodgkin lymphoma. CD38 is an ectoenzyme belonging to the family of nucleotide-metabolizing enzymes, involved in the scavenging of extracellular nucleotides. CD38 catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from NAD, leading to an increase in cytoplasmic  $\text{Ca}^{2+}$  concentration. CD38 is used as a disease marker for leukemias and myeloma and it is considered a negative prognostic marker for CLL (4;5). Moreover, recent evidence indicates that CD38 forms a complex molecular network delivering growth and survival signals in CLL cells. CD38 cooperates with chemokines and their receptors to influence cell migratory responses (11). These characteristics make CD38 an attractive target for CLL therapy. The use of an antibody such as DARA that specifically blocks CD38 might provide a new approach for interfering with deleterious growth circuits and for increasing the susceptibility of leukemic cells to conventional chemotherapy.

In this study, we have analyzed the potential therapeutic activity of DARA in CLL. DARA showed limited CDC activity in both primary CLL cells and CLL cell lines. Complement activation is strongly regulated by CRPs *in vivo* to prevent its uncontrolled amplification, including CD46, CD55, and CD59 that have been shown to mediate resistance to CDC induced by rituximab and less by ofatumumab (39). In fact, these proteins are overexpressed in a number of tumor types, and their upregulation has been postulated to contribute to mAb resistance *in vivo* (40). Our data show that CLL cell lines and primary cells display very high expression of the CRP, which may explain the limited sensitivity to DARA-mediated CDC *in vitro*.

Our results are the first to provide strong evidence that DARA induces lysis of CLL cells by  $\text{Fc}\gamma\text{R}$  mediated ADCC and ADCP through NK cells and macrophages, respectively. This cytotoxic effect is remarkable in CLL, where the mean ADCC induction was 35% at  $0.1\mu\text{g/mL}$ , being in the same range to those published for primary MM (19;32). In addition, ADCC does not correlate strictly with CD38 sABC in CLL cell lines or primary cells, indicating that at least in CLL the number of CD38 molecules on the cell surface may not be the only factor in driving Fc-mediated cytotoxicity of DARA. This observation suggests that other molecules within the immune synapse must control the extent of ADCC/ADCP by DARA. Activating receptors expressed on NK cells include  $\text{Fc}\gamma\text{RIIIA}$ , activating forms of Killer cell Ig-like receptors (KIRs) (KIR2DS and KIR3DS), NKG2D, and the Natural Cytotoxicity Receptors (NCR) called NKp30, NKp44, and NKp46 that are critical for optimal ADCC activity (41). NKG2D and NCRs are the most relevant receptors that stimulate responses to tumor target cells. In addition, inhibitory receptors counteract activating receptors as a means to tolerate mature NK cells. Thus, the overall make-up of these activating and inhibitory molecules on each individual CLL tumor cell may dictate the extent of ADCC by DARA (42). Along these lines, a recent study in MM have shown that blocking inhibitory KIRs with IPH2102, a human IgG4 monoclonal antibody that blocks the interaction of the three main inhibitory KIR receptors with their ligands, improves ADCC induced by daratumumab against MM cells (43).

Macrophages are tissue-resident immune cells that play a critical role not only in maintaining homeostasis and fighting infection but also in the progression of many

pathologies including cancer (44). Human macrophages express both activating and inhibitory Fc $\gamma$ R and are involved as most prominent effector cell populations in mAb-mediated tumor elimination *in vivo*. We have demonstrated that DARA induces phagocytosis of CLL by macrophages both *in vitro* and *in vivo*. In the *in vitro* model CLL cells and macrophages were co-cultured in the presence or absence of DARA. In the *in vivo* model, CLL cells were injected into the peritoneum of SCID *beige* mice, which are devoid of NK cells, but possess active macrophages in their peritoneal cavity.

Taken together these results provide evidence that anti-CD38 therapy with DARA may be relevant for CD38<sup>+</sup> CLL cases.

We next explored if these *in vitro* mechanisms translate to *in vivo* tumor growth inhibition. To accomplish this task we developed two approaches. First, we successfully developed a MEC2 tumor model that showed engraftment efficacy of 100% and systemic disease involving mostly lungs, kidney, ovaries, parathyroid glands, enlarged lymph nodes and BM in a portion of the mice. This model is clinically relevant for CLL with leukemic infiltrates in isolated organs similar to that reported in CLL patients (45), presenting aggressive disease or transformation to Richter's syndrome. In this model, treatment with relevant pharmacological doses of DARA efficiently prevented tumor progression and significantly prolonged survival. Mice treated with DARA showed long-term survival even though DARA dosing was stopped after 4 weeks and the antibody concentrations in the serum were minimal, suggesting that these mice were free of disease. Second, we developed a CLL-PDX model using NSG mice, to avoid CLL clearance by mouse NKs or phagocytes, and inoculating selected CLL enriched in NKs and monocytes to provide the system with Fc $\gamma$ R-bearing effector cells. In this model, DARA proved to reduce tumor burden in the mouse spleen, which constitutes the main infiltrated organ in this model (46). These results are the first to provide evidence that anti-CD38 therapy with DARA may be relevant for CD38<sup>+</sup> CLL.

As described in the introduction, DARA activity may extend beyond its effect on the tumor cells, as it shows immunomodulatory effects on CD38-expressing immunosuppressive regulatory T and B cells and myeloid-derived suppressor cells (23). We have also demonstrated additional activities of DARA besides ADCC and ADCP in CLL. DARA has the potential to counteract microenvironment-derived signaling in protective cancer niches, such as LN and BM. We have demonstrated that DARA interferes with *in vitro* cell migration and *in vivo* homing of CLL cells to spleen in NSG mice. Transendothelial migration and organ invasion of malignant cells require proteolytic degradation of the vascular basement membrane and extracellular matrix of lymphoid tissues and MMPs play a key role in these processes. MMP9 is the predominant MMP expressed in CLL and is physiologically regulated by CD49d/CD29 and CXCL12, playing a key role in cell invasion and transendothelial migration (47). Moreover, MMP9 correlates with advanced stage disease and poor patient survival (48). We have demonstrated that DARA significantly reduces CD49d/CD29-mediated adhesion of CLL cells to VCAM-1 and, more importantly, downregulates both constitutive and adhesion-induced MMP9 expression. Based on the prominent role of MMP9 on CLL cell invasion, our results indicate that DARA treatment may impede CLL tissue infiltration that leads to progressive disease. Thus, in the era of B-

cell receptor (BCR) kinase inhibitors, DARA immunotherapy opens a new horizon offering unique effects on tumor dissemination against CD38<sup>+</sup> CLL cases. In conclusion, our results support DARA as a novel therapeutic approach for CD38<sup>+</sup> CLL by not only inducing the classical FcγR-mediated cytotoxicity but also by harnessing microenvironment-derived survival signaling and blocking CLL dissemination to secondary lymphoid organs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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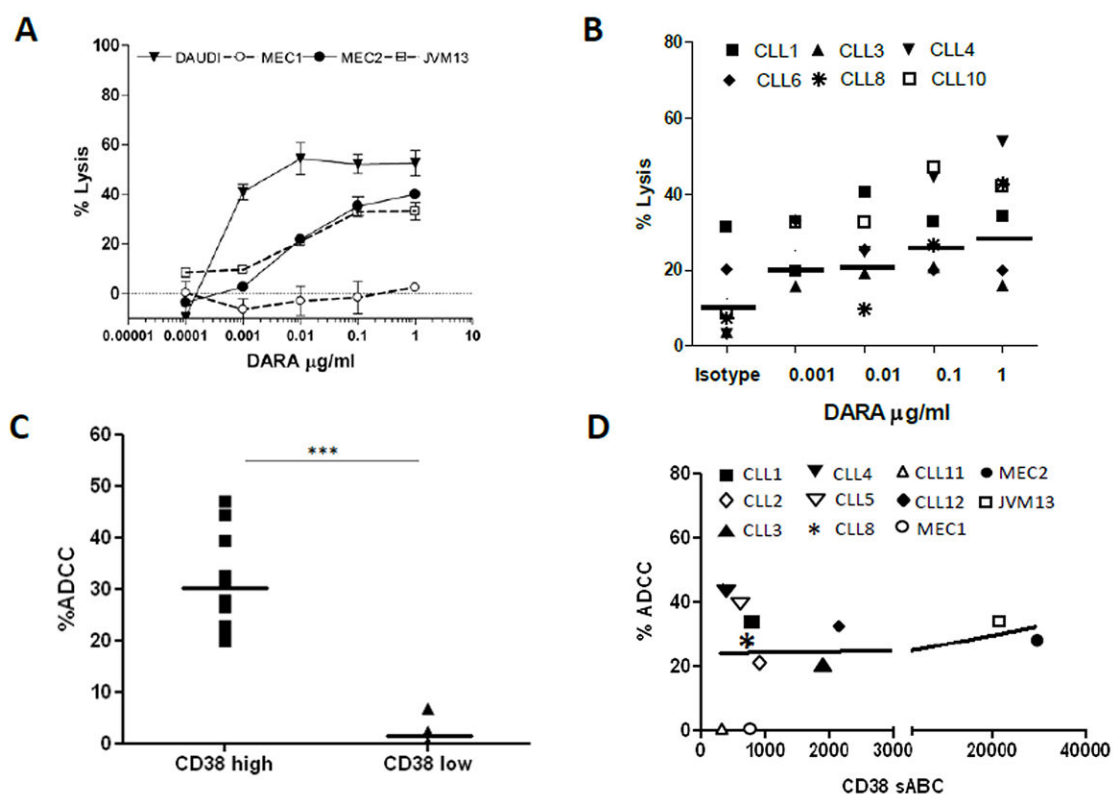
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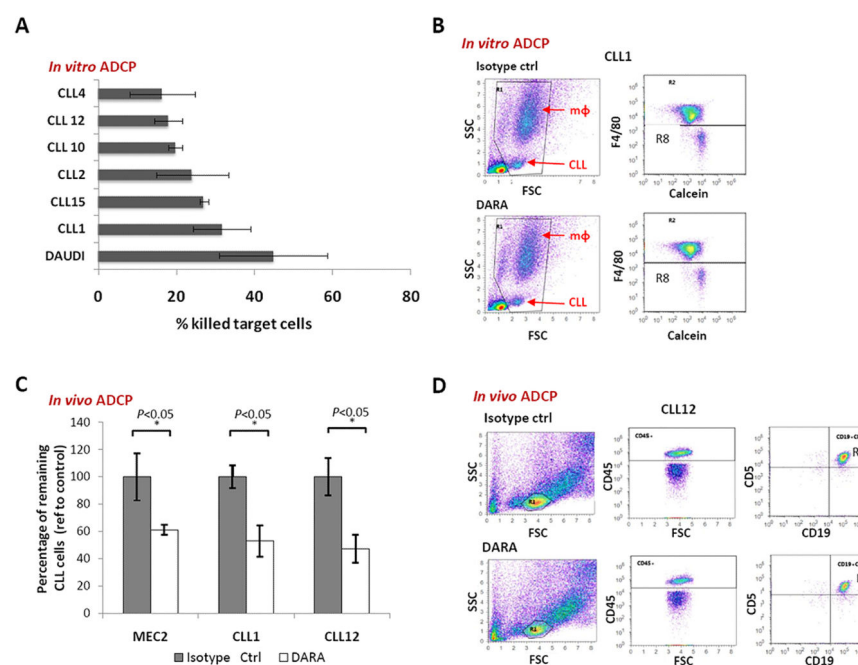
### TRANSLATIONAL RELEVANCE

Chronic lymphocytic leukemia (CLL) remains an incurable disease where high CD38 expression is associated with poor prognosis and identifies cells that are prone to proliferate. CD38 cooperates in migration, adhesion and invasion through its molecular association with CXCR4, MMP9 and CD49d. The human anti-CD38 monoclonal antibody daratumumab has shown efficient cell killing and a good safety profile in clinical trials in multiple myeloma. Here, we demonstrate that daratumumab also exerts significant cytotoxicity against patient-derived CLL cells, via ADCC and ADCP *in vitro* and *in vivo*. Furthermore, daratumumab interferes with CD38 signaling and reduces CLL cell adhesion, migration and homing. Moreover, daratumumab shows therapeutic activity in two mouse models. Thus, daratumumab improves overall survival in a systemic CD38<sup>+</sup> MEC2 cell line mouse model and reduces tumor burden in CLL-patient derived xenografts. These results provide scientific rationale for the clinical development of daratumumab in poor prognosis CD38<sup>+</sup>CLL.



**Figure 1. Daratumumab induces ADCC in the presence of external effectors**

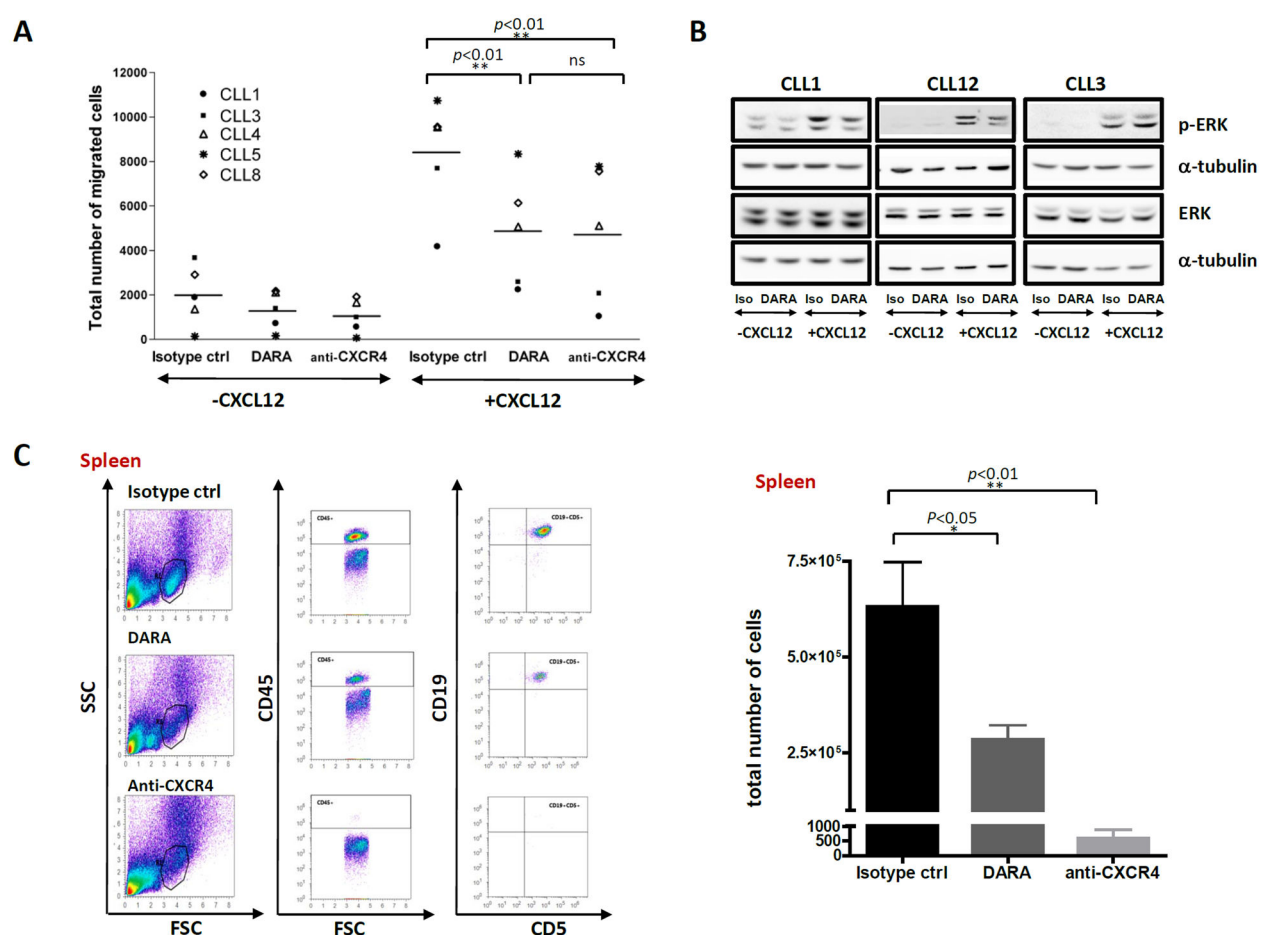
Daudi cells, CLL cell lines, both CD38<sup>high</sup> (MEC2 and JVM13) and CD38<sup>low</sup> (MEC1) (A) and primary CLL cells (B) were treated with increasing daratumumab (DARA) doses (0.0001-1  $\mu\text{g/ml}$ ) in the presence of PBMC from healthy donors at a E:T ratio of 50:1 for 4 hours. Viability was then evaluated by calcein release assay. In panel B, ADCC induced by isotype control at the maximal dose of 1  $\mu\text{g/ml}$  is also depicted and the horizontal line represents the mean lysis. (C) ADCC induction by daratumumab (0.1  $\mu\text{g/ml}$ ) in CD38 high (>30%) vs CD38 low (<30%) CLL primary cases. (D) The number of surface antibodies bound per cell (sABC) of CD38 was quantified in primary CLL cells and cell lines and plotted for correlation with ADCC induction by DARA (cell lines 0.1  $\mu\text{g/ml}$  and primary CLL 0.01  $\mu\text{g/ml}$ ).



**Figure 2. Daratumumab induces ADCP *in vitro* and *in vivo***

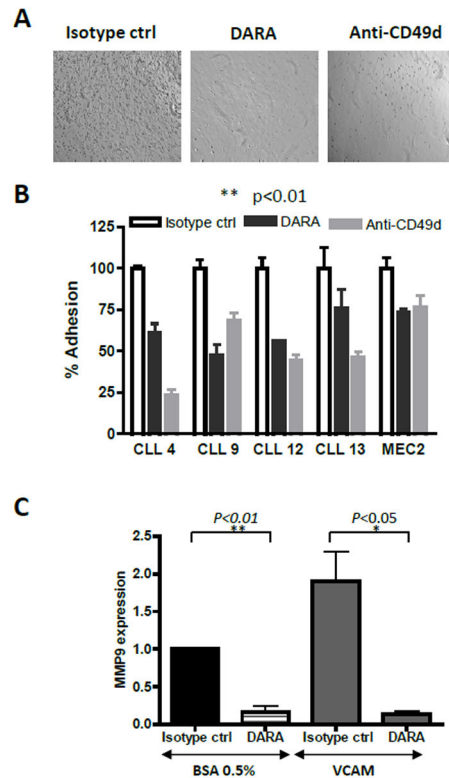
(A) CLL cells were treated in triplicates with a fixed concentration (1 $\mu$ g/mL) of DARA or isotype control in the presence of mouse macrophages at a E:T ratio of 1:1, for 4 hours. Percentage of killed target cells was calculated by flow cytometry. CLL cells were identified as Calcein<sup>+</sup> F4/80<sup>-</sup> and the percentage of killing by DARA was calculated according to the formula included in materials and methods. Daudi cell line was used as positive control. (B) Representative flow cytometry plots of *in vitro* ADCP. CLL cells and macrophages (mφ) are clearly seen in the FSC/SSC density plot. The number of cells in the R8 gate was used to calculate the percentage of killed tumor cells. (C) *In vivo* phagocytosis was evaluated in SCID *beige* mice (n=3-5 per group) that were inoculated *i.p.* with primary CLL cells or MEC2 cell line (2 $\times$ 10<sup>7</sup> cells per mouse) and subsequently treated *i.p.* with one dose of 20 mg/kg of DARA or isotype control. The mice were sacrificed 48 hours later and cells from the peritoneum recovered and counted by flow cytometry as huCD45<sup>+</sup>CD19<sup>+</sup>CD5<sup>+</sup> for primary CLL cells and CD45<sup>+</sup>CD19<sup>+</sup>CD5<sup>-</sup> for MEC2 cells. The percentage of residual leukemia cells after DARA treatment is plotted where the total number of cells remaining treated with the isotype control was set to 100% (p<0.05, unpaired t-test). (D) Representative flow cytometry plots where the R3 gate was used to calculate the percentage of killed tumor cells. The gating strategy started with cells in FSC/SSC (R1), then gating on CD45<sup>+</sup>, and finally CD19<sup>+</sup>CD5<sup>+</sup>.





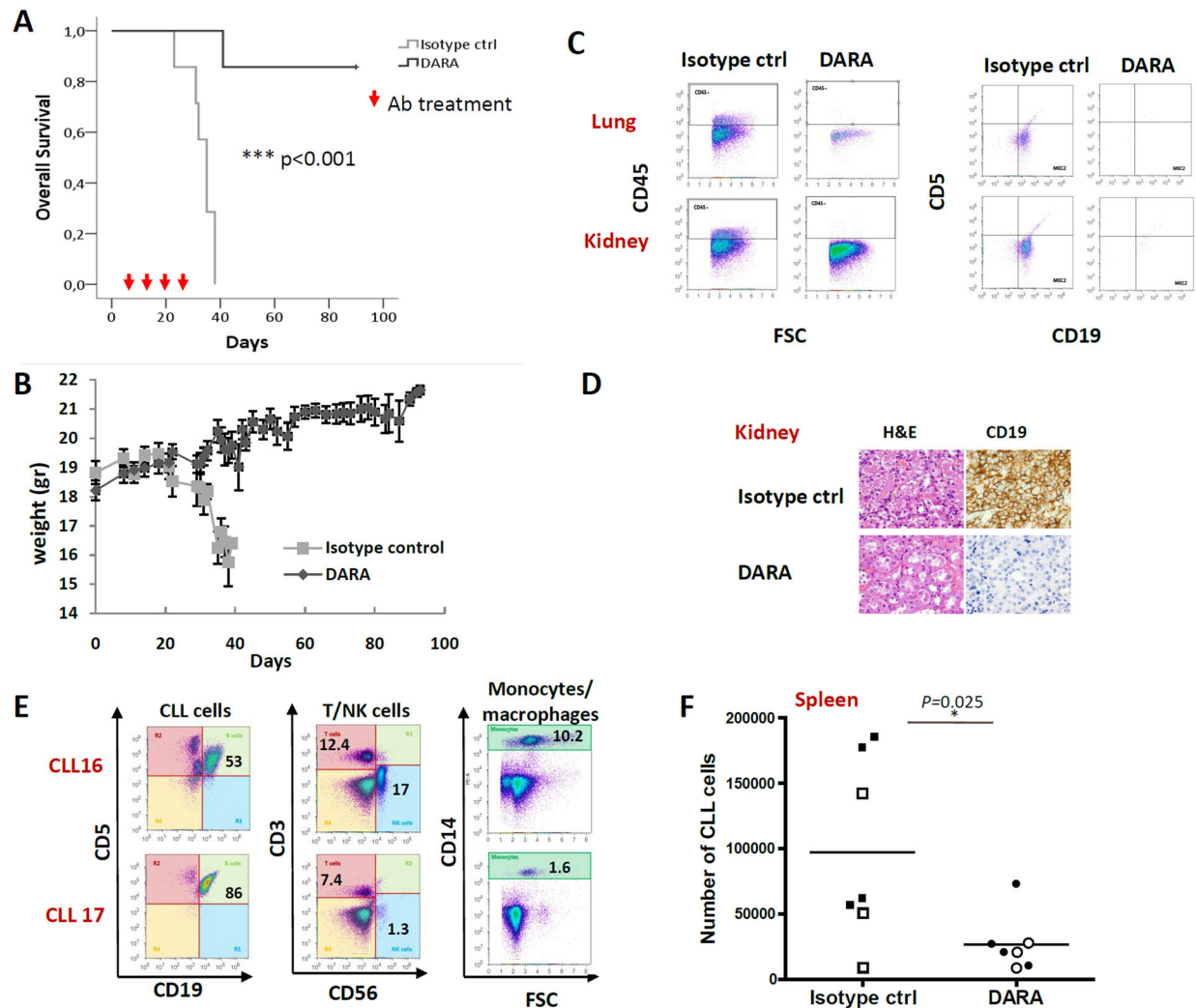
**Figure 3. Daratumumab interferes with CLL cell migration and *in vivo* homing**

(A) CLL cells were preincubated with the antibodies for 30 min at 4°C (30 µg/mL for isotype control and DARA and 25 µg/mL anti-CXCR4) and then assayed for migration in a CXCL12 gradient. After 4h, CLL cells (CD19<sup>+</sup> CD5<sup>+</sup>) in the lower chamber were counted in triplicates in a flow cytometer at fix flow rate. Total number of cells is graphed for representative CLL patients (n=5). Statistical differences between groups were assessed by paired t-test. (B) Western blot analysis of ERK activation after stimulation of CLL cells for 5 minutes with CXCL12 (200 ng/mL). Before stimulation, cells were serum starved for 2h and pretreated for 30 min with the corresponding antibodies (30 µg/mL). (C-D) *In vivo* homing was assessed by *iv* inoculation of fresh CLL cells via tail vein in NSG mice, previously pretreated with the corresponding antibodies (10 mg/kg, n=4 mice/group). After 20h, cells were recovered from spleen, labeled with huCD45/CD19/CD5 and counted in a flow cytometer. Representative density plots for each treatment are shown (C). The gating strategy started with cells in FSC/SSC (R1), then gating on huCD45<sup>+</sup>, and finally huCD19<sup>+</sup> CD5<sup>+</sup>. (D) Total number of huCD45<sup>+</sup>CD19<sup>+</sup>CD5<sup>+</sup> recovered from the spleen is plotted. Statistical differences between groups were assessed by unpaired t-test.



**Figure 4. Daratumumab hampers CLL adhesion to VCAM**

Calcein-labeled primary CLL and MEC2 cells were pre-incubated with the corresponding antibodies (30µg/mL) and left to adhere for 30 min to plates pre-coated with VCAM-1 or BSA (non-specific adhesion). Anti-CD49d was used as positive control for inhibition of adhesion. Non-adhered cells were removed by extensive washing. (A) Representative phase-contrast microscopy field images (×100) from adhesion to VCAM-1 of CLL9. (B) Adhered cells were then lysed and supernatants analyzed in a fluorimeter. Percentage of VCAM-1 adhesion is expressed normalized to isotype control and after subtraction of BSA non-specific adhesion ( $p<0.01$ , one way-ANOVA comparing the three groups together). (C) RT-PCR for MMP9 was performed on the adhered cells to VCAM, using mGUS as endogenous control. (n=3, CLL4, CLL12 and CLL13) Expression levels for each sample are normalized to the corresponding isotype control and adhesion to BSA. \*\* =  $p<0.01$  and \* =  $p<0.05$ , unpaired t-test).



**Figure 5. In vivo efficacy of daratumumab**

(A) Kaplan-Meier survival curves for MEC2 systemic mouse xenografts. Mice received DARA (n=7) or isotype control (n=6) weekly for four weeks, starting one week after cell inoculation. Then, mice were monitored twice a week for any sign of disease and sacrificed when body weight decreased 15-20%. All control mice had to be sacrificed between days 23-38. One DARA-treated mouse became ill and was sacrificed on day 41. (B) Body weight changes in the isotype and DARA treated mice. (C) Cells from lungs and kidneys were isolated and labeled with huCD45/CD19/CD5. The presence of MEC2 cells was evaluated by CD19<sup>+</sup>CD5<sup>+</sup> (right panel) cells in the CD45<sup>+</sup> population (left panel). Representative flow cytometry density plots for one mouse of each cohort sacrificed at day 30 are shown. (D) IHC staining of H&E and CD19 of kidneys from isotype and DARA treated mice (magnification x400). (E) Density plots showing the percentage of CLL cells (CD19<sup>+</sup>CD5<sup>+</sup>), T cells (CD3<sup>+</sup>CD56<sup>-</sup>), NK cells (CD3<sup>-</sup>CD56<sup>+</sup>) and monocytes (CD14<sup>+</sup>). (F) Total number of huCD45<sup>+</sup>CD19<sup>+</sup>CD5<sup>+</sup> recovered from the spleen of isotype ctrl and DARA

treated groups (CLL16: open symbols; CLL17: closed symbols). Statistical differences between groups were assessed by unpaired t-test.

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**Table 1**

CLL patient characteristics

Study label	Gender <sup>1</sup>	Binet/Rai stage	%tumor cells <sup>2</sup>	IgVH status <sup>3</sup>	CD38 <sup>4</sup>	CD38 sABC <sup>5</sup>	CD46 <sup>4</sup>	CD55 <sup>4</sup>	CD59 <sup>4</sup>	CD49d <sup>4</sup>	%CD38 <sup>6</sup>	%ADCC <sup>7</sup>
CLL1	M	C/IV	95	nd	98	816	98	91	9	4	nd	32.7
CLL2	M	A/I	96	UM	63	897	889	88	96	6	6.4	20.8
CLL3	M	C/IV	86	UM	80	1875	90	88	79	68	8.4	20.8
CLL4	M	A/I	97	UM	31	355	92	90	75	79	nd	44.5
CLL5	M	B/II	97	UM	44	562	92	90	97	1	0.1	39.4
CLL6	F	C/III	86	M	50	nd	nd	nd	nd	nd	25.1	20
CLL7	M	C/III	96	UM	2	nd	98	99	90	0.7	5.6	0.2
CLL8	M	A/0	95	M	53	736	93	94	83	5	3.4	26.6
CLL9	M	A/I	82	nd	73	nd	98	97	98	63	0.9	22.9
CLL10	M	B/III	93	M	64	nd	92	91	94	100	25.6	47
CLL11	M	A/0	97	M	0.5	294	95	94	98	0.1	0	0
CLL12	F	A/III	94	UM	95	2132	97	87	90	80	17.4	31.4
CLL13	M	A/0	60	nd	55	nd	99	100	99	98	10.4	nd
CLL14	M	B/II	98	UM	66	nd	97	93	86	nd	nd	27.9
CLL15	F	A/I	86	UM	71	1531	96	98	88	83	nd	27.2
CLL16	M	A/I	53	nd	75	nd	98	94	96	95	23.2	38.1
CLL17	M	A/0	85	UM	83	nd	99	98	96	nd	0.3	nd
CLL18	M	A/0	91	UM	70	nd	98	98	97	92	0	22

<sup>1</sup>M: male; F: female

<sup>2</sup>Percentage of tumor cells assessed by flow cytometry based on CD19<sup>+</sup>CD5<sup>+</sup>CD23<sup>+</sup> cells;

<sup>3</sup>Determined by direct sequencing. UM: unmutated, sequence homology > 98%. M: mutated, sequence homology < 98%. nd: not determined.

<sup>4</sup>Percentage of positive cells for CD38, CD46, CD55, CD59 and CD49d determined by flow cytometry in CD19<sup>+</sup>CD5<sup>+</sup> population, referred to isotype control. CD38 was considered positive when the percentage of positive cells exceeded 30%

<sup>5</sup>sABC: number of surface antibodies bound per cell evaluated by QuantiBRITE™ CD38-PE.

<sup>6</sup>Percentage of CDC induction at 10 µg/mL DARA.

Percentage of ADCC induction at 0.1 µg/mL DARA.

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